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Urinary mutagenicity as a biomarker in workers exposed to benzidine: correlation with urinary metabolites and urothelial DNA adducts

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Urinary mutagenicity has been used in occupational and epidemiological studies for over two decades as a cost-effective, general biomarker of exposure to genotoxic agents. However, few studies have compared urinary mutagenicity to additional biomarkers determined among low- and high-exposed groups. To address this issue, we evaluated the relationship between urinary mutagenicity and other types of biomarkers in a cross-sectional study involving 15 workers exposed to the urinary bladder carcinogen benzidine (BZ, high exposure), 15 workers exposed to BZ-dyes (low exposure), and 13 unexposed controls in Ahmedabad, India. Urinary organics were extracted by C18/methanol and evaluated for mutagenicity in the presence of S9 in the *Salmonella* strain YG1024, which is a frameshift strain that overproduces acetyltransferase. The results were compared to biomarker data reported recently from the same urine samples (Rothman *et al.*, *Proc. Natl Acad. Sci. USA*, 93, 5084–5089, 1996) that included a metabolite biomarker (the sum of the urinary levels of BZ + *N*-acetylbenzidine + *N,N'*-diacetylbenzidine) and a DNA adduct biomarker [a presumptive *N*-(3'-phosphodeoxyguanosin-8-yl)-*N'*-acetylbenzidine (C8dG-ABZ) DNA adduct in exfoliated urothelial cells]. The mean ± SE urinary mutagenicity (revertants/μmol of creatinine) of the low-exposure (BZ-dye) workers was 8.2 ± 2.4, which was significantly different from the mean of the controls (2.8 ± 0.7, $P = 0.04$) as was that of the mean of the high-exposure (BZ) workers (123.2 ± 26.1, $P < 0.0001$). Urinary mutagenicity showed strong, positive correlations with urinary metabolites ($r = 0.88$, $P < 0.0001$) and the level of the presumptive C8dG-ABZ urothelial DNA adduct ($r = 0.59$, $P = 0.0006$). A strong association was found between tobacco use (bidi smoking) and urinary mutagenicity among the controls ($r = 0.68$, $P = 0.01$) but not among the exposed workers ($r = 0.18$, $P = 0.11$). This study confirms the ability of a biomarker such as urinary mutagenicity to detect low-dose exposures, identify additional genotoxic exposures among the controls, and correlate strongly with urinary metabolites and DNA adducts in the target tissue (urinary bladder epithelia) in humans.

*Abbreviations: BZ, benzidine; ABZ, *N*-acetylbenzidine; DABZ, *N,N'*-diacetylbenzidine.

Introduction

Biomarkers that reflect the spectrum of cancer pathogenesis from exposure to disease are important components of human population monitoring and cancer epidemiology studies (1–3). Some biomarkers are highly specific to the exposure or the resulting disease, such as the measurement of urinary metabolites and DNA adducts resulting from aflatoxin B1 exposure (4). Others may be more general, such as the measurement of chromosomal aberrations (5). An important example of this latter category of biomarkers is urinary mutagenicity, which has been used for more than 20 years to evaluate life-style and occupational exposure to mutagens and potential carcinogens (6,7).

Urinary mutagenicity has been demonstrated among people who smoke cigarettes (8), are exposed to mutagenic drugs (9–12), or eat fried meats (13–15). It has also been used as a biomarker of exposure in various occupational settings (16), including workers in industries such as rubber (17–19), ink and pharmaceutical (20), textiles (21), and sewage-treatment (22); as well as among asphalt pavers (23) and people exposed to trinitrotoluene (24). Although urinary mutagenicity is non-specific in that it does not identify the various mutagens in the urine, it has the advantage of integrating the mixture of mutagenic compounds in the urine and detecting the sum of these components, without the need for analytical methods to identify each mutagenic compound.

Some studies have found significant, positive correlations between urinary mutagenicity and other general biomarkers, such as sister chromatid exchanges in blood cells or urinary excretion of thioethers (23,25–27). Urinary mutagenicity has also been compared to phase I and phase II metabolic genotype and/or phenotype among smokers and textile workers (21,27–29). However, as reviewed below, only a few studies have correlated urinary mutagenicity with a biomarker that reflected a specific exposure.

Urinary mutagenicity has been found to correlate with the levels of urinary metabolites in people exposed to trinitrotoluene (24), metronidazole (30), cigarette smoke (28,31–33), and coal tar (34,35); and with the levels of a 4-aminobiphenyl-hemoglobin adduct (28,36) and DNA adducts among cigarette smokers (32,36). A recent cross-sectional study reported by Rothman *et al.* (37) has provided the opportunity to extend these observations by comparing urinary mutagenicity both to urinary metabolites and to DNA adducts among 30 workers exposed to benzidine (BZ*) or BZ-based dyes and 15 unexposed controls from Ahmedabad, India. This cross-sectional study can be viewed as a model system in which a variety of biomarkers can be compared among a group of workers who were exposed to potent mutagenic/carcinogenic compounds that posed a well-documented risk for bladder cancer. If the examined biomarkers could not distinguish collectively among the variously exposed groups of workers exposed to high levels of potent carcinogens, then the use of these biomarkers among people exposed to lower levels or to less carcinogenic compounds would be questionable. As described below, the present report compares urinary mutagenicity among these workers to two biomarkers that are more specific to BZ exposure.

Various aromatic amines used in the dye industry, especially BZ and BZ-based dyes, were identified in the 1970s as carcinogens for the human urinary bladder (38–40). BZ is metabolized to a variety of acetylated and/or hydroxylated forms, such as *N*-acetylbenzidine (ABZ) and *N,N'*-diacetylbenzidine (DABZ) (41,42). BZ and its acetylated metabolites are mutagenic (41); rats exposed to BZ produce mutagenic urine (43–45); and BZ is metabolized by rat liver *in vitro* to form a specific DNA adduct, C8dG-ABZ (46), which has also been identified presumptively as the predominant DNA adduct in liver cells of rodents exposed *in vivo* and *in vitro* to BZ (47–50).

In the cross-sectional study described above (37), a strong, positive correlation was found between the sum of the concentrations of BZ + ABZ + DABZ in the urine and the level of a presumptive C8dG-ABZ DNA adduct in exfoliated urothelial cells. However, the *N*-acetyltransferase (NAT1 and NAT2) status of the workers was not associated with the levels of urinary metabolites or urothelial DNA adducts (37), which is consistent with a case-control study in which NAT2 status was not associated with bladder cancer risk in an occupational cohort of workers exposed to BZ (51,52). In addition, the glutathione *S*-transferase M1 (*GSTM1*)-null genotype also had no impact on the risk for BZ-associated bladder cancer or on the levels of BZ-associated urothelial cell DNA adducts, urinary metabolites, or urinary mutagenicity (53). Here we show the mutagenic potencies of the urines from the individual workers from this cross-sectional study and compare these to (i) the sum of the urinary concentration of BZ + ABZ + DABZ in each worker and (ii) the level of the presumptive C8dG-ABZ DNA adduct in the exfoliated urothelial cells of the workers.

Because BZ must be acetylated and *N*-hydroxylated to be mutagenic, we have evaluated urinary mutagenicity among these workers with strain YG1024 of *Salmonella*, which overproduces acetyltransferase, and the experiments were performed in the presence of rat liver S9 to provide *N*-hydroxylation and additional acetylation activity. The results are compared with those from studies of cigarette smokers and workers from other occupations. We discuss the implications of the results regarding the role of acetylation in the metabolism of BZ by humans *in vivo*. The general utility of urinary mutagenicity as a biomarker and its relationship to other biomarkers, such as urinary metabolites and DNA adducts, are also evaluated relative to BZ and other exposures studied in the literature, such as cigarette smoke.

Materials and methods

Cross-sectional study

The field phase of this study was conducted in 1993 as described previously (37) under the auspices of the National Institute of Occupational Health in Ahmedabad, India. After the study was explained to the subjects, informed consent was obtained. Fifteen workers exposed to BZ were enrolled from four factories that manufactured benzidine dihydrochloride. Eighteen workers exposed to BZ-based dyes were enrolled from three factories that manufactured primarily Direct Black 38, using benzidine dihydrochloride as a starting product. Because three of these 18 workers were subsequently found to have no detectable urinary BZ or BZ metabolites, they were omitted from the analysis (37). Fifteen control workers were enrolled from a building construction company and were frequency-matched to the exposed workers by age (5-year groups) and current smoking status. The factories that manufactured BZ and BZ-based dyes were dusty, and workers had extensive dermal and respiratory contact with these compounds. BZ and BZ-dye manufacturing and use were banned in India in 1994, and these factories were subsequently closed.

All subjects were male and were administered a questionnaire by trained interviewers to collect information on occupational history, medical history, current and lifelong tobacco use, and usual alcohol intake. Subjects provided post-workshift urine samples that were processed, stored, and shipped to a US National Cancer Institute biorepository as described previously (37). Urine samples were collected in a consistent manner, with subjects voiding two-thirds of the way through the workshift and then providing a spot urine sample immediately at the end of the workshift. The mutagenicity and metabolite analyses were performed with blind-coded samples with respect to the exposure status of the subjects.

Mutagenicity assays

Urinary mutagenicity was evaluated at the US Environmental Protection Agency. Frozen urine was thawed, and 37 ml were passed through two, 6-ml C18 resin columns (J.T.Baker, Phillipsburg, NJ) stacked in tandem with each containing 500 mg of C18. The organics were eluted with 10 ml of methanol and solvent exchanged into 185 μl of dimethyl sulfoxide (DMSO, Burdick and Jackson, Muskegon, MI) to make a 200× concentrate. Preliminary experiments comparing the extraction efficiencies of C18 and cyanopropyl (CN) columns using benzidine in water showed that either resin gave ~100% mass recovery (data not shown). Based on prior experience and availability, C18 was selected for this study.

Dilutions of the organic extracts in DMSO were evaluated in the standard *Salmonella* plate-incorporation mutagenicity assay (54) using strain YG1024, which is a derivative of the frameshift tester strain TA98 (*hisD3052*, *uvrB*, *rfa*, pKM101) that contains elevated levels of acetyltransferase activity (55). All samples were evaluated in the presence of aroclor-induced male Sprague–Dawley rat liver S9 (2 mg of S9 protein/plate) that was prepared as described (54). The doses of urine extracts tested were 0.2, 0.5, 1, 1.5, 2.5, 5, 10, and 15 ml equivalents (ml-eq) per plate. With these doses, one plate/dose and one experiment were possible with the starting sample volume of 37 ml. Revertant (rev) colonies were counted after 3 days of incubation at 37°C, and mutagenic potencies (rev/ml-eq) were calculated from the slope of the regression over the linear portion of the dose–response curves. Half of the samples was tested in one experiment, and the other half in a second experiment 4 days later. Controls consisted of DMSO (100 μl/plate), C18 resin blanks in which 37 ml of glass-distilled deionized water instead of urine were passed through the columns (15 ml eq/plate), and 2-aminoanthracene at 500 μg/plate. After adjustment for creatinine levels, the results were expressed as rev/μmol of creatinine.

BZ + ABZ + DABZ analysis

The concentrations of free BZ, ABZ, and DABZ in C18/methanol extracts of the urine samples were determined at the VA Medical Center and St Louis University School of Medicine as described previously (56). After extraction, the organics were derivatized with pentafluoropropionic anhydride and detected by capillary GC/negative chemical ion mass spectrometry. Results were expressed as the sum of the concentrations of the three compounds in the urine (ng/ml); after adjusting for creatinine levels, the results were expressed as ng/μmol of creatinine.

Urothelial cell DNA adduct analysis

The processing of the urine, isolation of urothelial cells, DNA extraction, and ^{32}P-postlabeling were performed at the University of Cincinnati as described previously (37). Chromatographic conditions detected C8dG-ABZ (46), C8dG-BZ (46,56,57), and the major smoking-associated DNA adducts, such as C8dG-4-aminobiphenyl (36). Adduct levels were quantified as described previously (36) and expressed as relative adduct labeling (RAL, calculated as $\text{c.p.m.}_{\text{adducts}}/\text{c.p.m.}_{\text{unadducted nucleotides}}) \times 10^9$. Values are the mean of 2–6 independent replications. The RAL of a urothelial cell DNA adduct that co-chromatographed with the synthetic C8dG-ABZ standard was labeled 'adduct 4' in a previous study (37). In the present study, we refer to adduct 4 as the presumptive C8dG-ABZ DNA adduct, and the level of this adduct is compared with the level of urinary mutagenicity in the workers.

Statistical analyses

Overall group differences in demographic characteristics were tested by the Kruskall–Wallis test. Urinary mutagenicity, urothelial cell DNA adducts, and urinary BZ and its metabolites in the control group were compared to values

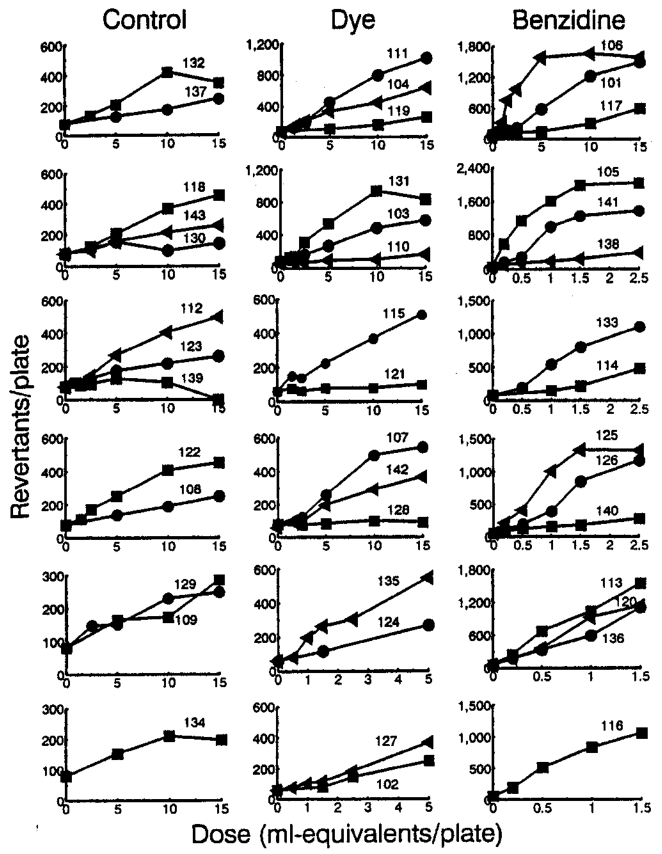

Fig. 1. Mutagenicity dose–response curves of C18/methanol organic extracts of urines in strain YG1024 of *Salmonella* in the presence of aroclor-induced rat liver S9. The subject number is noted on each curve.

in the BZ-dye and BZ workers using the Wilcoxon rank sum test. Biomarker data were then normalized with a natural logarithmic transformation. Correlations between biomarker values were calculated using Pearson's correlation test. Linear regression was used to evaluate the role of tobacco use, age, alcohol, and urinary BZ and its metabolites on each outcome. Two-sided P-values were calculated throughout; P-values <0.05 were considered significant.

Results

Demographics and exposure assessment

As noted previously (37), three populations were selected to have a range of exposures to BZ (none, low, and high) based on the materials and chemicals with which they worked and the working conditions in various factories. Exposure assessment determined by the urinary concentration of BZ + ABZ + DABZ (ng/μmol creatinine, mean ± SE) confirmed that the controls had no BZ exposure (0.0 ± 0.0), the BZ-dye workers had low exposure (3.2 ± 1.6), and the BZ workers had high exposure (46.5 ± 7.9) (37). All subjects were male and had negligible cigarette use. Other demographic characteristics were similar in each group, with the mean ± SD age being 22.4 ± 3.2 (controls), 23.7 ± 3.8 (BZ-dye), and 26.5 ± 5.1 years (BZ). The mean ± SD number of bidi (a type of cigarette) used per day was 2.5 ± 4.3 (controls), 3.6 ± 4.7 (BZ-dye), and 2.2 ± 3.7 (BZ). There were no significant overall or group-specific differences for age, bidi use or alcohol intake.

There was sufficient urine available for mutagenesis studies from 13 of the 15 control subjects. Because three of the 18 BZ-dye workers had no detectable levels of BZ, ABZ, or DABZ in their urine (37), they were eliminated from the

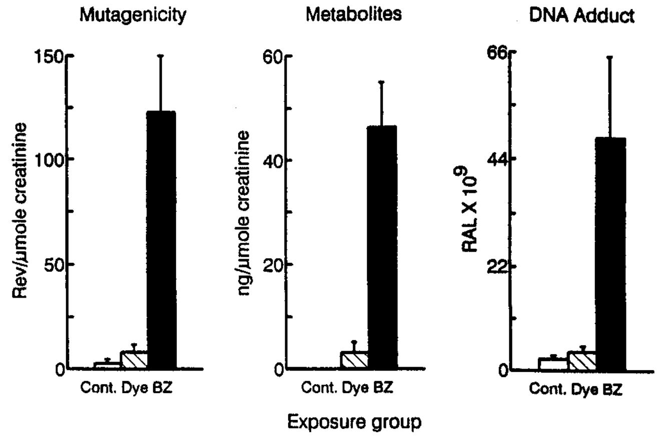

Fig. 2. Arithmetic mean ± SE of the urinary mutagenicity, the urinary concentrations of BZ + ABZ + DABZ, and the levels of the presumptive C8dG-ABZ DNA adduct in exfoliated urothelial cells from the three groups of workers. Mutagenicity data were calculated after adjustment for urinary creatinine concentrations; other data are from Reference (37). The ability of the biomarkers to distinguish between the occupational groups was evaluated by the Wilcoxon rank sum test. The P-values for control (n = 13) versus BZ-dye workers (n = 15) for the three biomarkers were mutagenicity (0.04), metabolites (<0.0001), and DNA adduct (0.33); the P-values were <0.0001 for all three biomarkers for control versus BZ workers (n = 15). The two BZ-dye workers who had non-mutagenic urine (subjects 121 and 128) were assigned mutagenicity values of 2.75 rev/μmol of creatinine. This value was determined by first identifying the lowest mutagenicity value in rev/ml that was greater than 0 among all the subjects, dividing that value by two, and then adjusting it for urinary creatinine concentration.

analysis presented here. Thus, this study evaluated urinary mutagenicity in 43 subjects: 13 controls, 15 BZ-dye workers, and 15 BZ workers.

Urinary mutagenicity

Figure 1 shows the mutagenicity dose–response curves for the 43 workers grouped according to occupation. The dose–response curves from most of the controls and BZ-dye workers reached a plateau or declined at 10 or 15 ml-eq/plate; however, this occurred at 2.5 ml-eq for most of the samples from the BZ workers. The urines from two of the controls (subjects 130 and 139) and from two of the BZ-dye workers (subjects 121 and 128) did not produce a doubling of the mutant yield (rev/plate) at the maximum dose tested (15 ml-eq/plate) relative to the background mutant yield. Thus, these four samples were considered non-mutagenic. For the remaining samples, the doses used permitted the generation of dose–response curves with linear portions from which mutagenic potencies (rev/ml-eq) were calculated. Mutagenic potencies ranged from essentially zero among some of the control and BZ-dye workers, to >1000 rev/ml-eq among the BZ workers.

Most of the control subjects had mutagenic urine, with potencies generally >10 rev/ml-eq. Because smoking can contribute to urinary mutagenicity, we examined the impact of bidi-use among both the controls and exposed workers. Among the 13 controls, bidi-use was strongly associated with urinary mutagenicity (Pearson r = 0.68, P = 0.01), whereas this association was weakened substantially among the 30 exposed workers after adjusting for urinary BZ-associated metabolites (r = 0.18, P = 0.11). The small, but positive r value for the exposed workers suggests that bidi-use may have contributed slightly, although not significantly, to the urinary mutagenicity of the exposed workers.

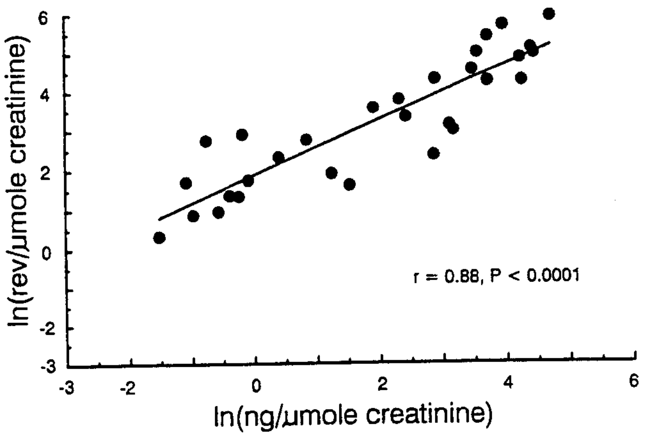

Fig. 3. Correlation (Pearson r) between urinary mutagenicity (rev/µmol of creatinine) and urinary concentrations of BZ + ABZ + DABZ (ng/µmol creatinine) of the exposed workers (n = 30). Mutagenicity data were calculated after adjustment for urinary creatinine concentrations and ln-normalized; other data are from reference 37.

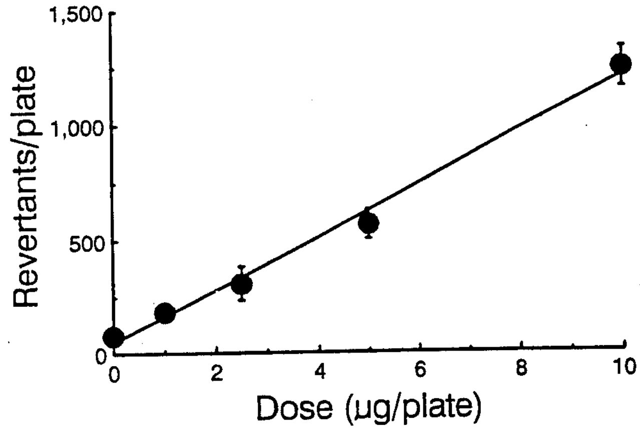

Fig. 4. Mutagenicity dose–response curve of BZ in strain YG1024 of *Salmonella* in the presence of aroclor-induced rat liver S9. Results are the mean ± SE of four plates/dose (from two experiments, each in duplicate). The mutagenic potency calculated from the slope of the line is 116 rev/µg.

Comparison of urinary mutagenicity with urinary metabolites

The extent to which urinary mutagenicity characterized the exposure of the workers is illustrated in Figure 2, which shows the means of the urinary mutagenicity data for the three groups of workers along with the corresponding values from the metabolite and DNA adduct analyses (37). The mean ± SE urinary mutagenicity (rev/µmol creatinine) of the controls, BZ-dye, and BZ workers was 2.8 ± 0.7, 8.2 ± 2.4 and 123.2 ± 26.1, respectively. The corresponding means for the urinary metabolites (ng/µmol creatinine) were 0 (controls), 3.2 ± 1.6 (BZ-dye), and 46.5 ± 7.9 (BZ). The two biomarkers (urinary mutagenicity and metabolites) increase across the three exposure groups. The mean urinary mutagenicity of the BZ-dye workers was three times greater than that of the controls (P = 0.04, Wilcoxon rank sum test). The urinary mutagenicity of the BZ workers was 44 times greater than the controls ($P <$ 0.0001). The two exposed groups showed 15-fold differences in both urinary mutagenicity and urinary metabolite levels.

In order to evaluate how well the urinary mutagenicity and metabolite data paralleled each other, the correlation between them among workers with confirmed BZ exposure was determined using the data from 15 BZ-dye workers and 15 BZ workers. As shown in Figure 3, a strong, positive correlation was obtained between urinary mutagenic potency and the level of urinary metabolites (Pearson r = 0.88, $P <$ 0.0001). This provides supporting evidence that the BZ-associated urinary mutagenesis was, in fact, due to the presence of BZ and its metabolites in the urine.

Based on the slope of the line in Figure 3, the mutagenic potency of the workers' urine in terms of the detected amounts of BZ + ABZ + DABZ in the urine was ~2 rev/ng. Under the conditions of the mutagenicity assay used here, BZ itself is 1/20th as potent as this estimated potency of the workers' urine, producing ~0.1 rev/ng (Figure 4). As discussed later, this estimated mutagenic potency of the extracted organics in the urine samples is probably an overestimate because there are probably other mutagenic forms of BZ in the urines besides the three compounds detected.

Comparison of urinary mutagenicity with urothelial DNA adducts

As illustrated in Figure 2, the mean ± SE of the presumptive C8dG-ABZ DNA adduct in urothelial cells among the controls, BZ-dye and BZ workers was 2.4 ± 0.6, 4.0 ± 0.9 and 48.2 ± 15.5, respectively. The mean urinary mutagenicity went up in parallel with the DNA adduct levels across the three exposure groups. The mean DNA adduct level among the BZ-dye workers was 1.7 times greater than the controls; the DNA adduct levels were not significantly different between the controls and the BZ-dye workers (P = 0.33). However, the mean DNA adduct level of the BZ workers was 20 times greater than the controls, which was significantly different (P < 0.0001), and there was a 12-fold difference between the two exposed groups.

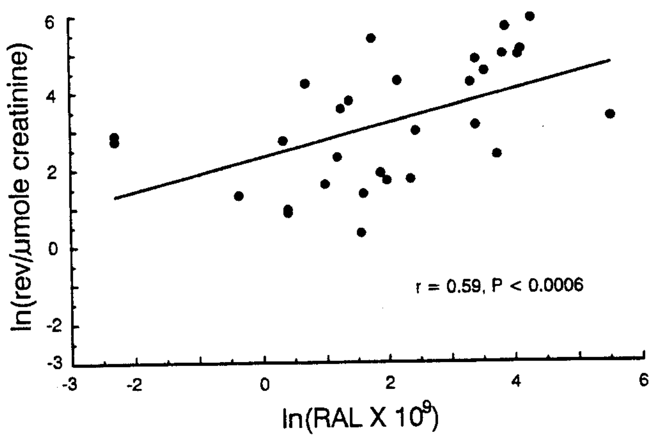

Fig. 5. Correlation (Pearson r) between urinary mutagenicity (rev/µmol of creatinine) and the RAL of the presumptive C8dG-ABZ DNA adduct in exfoliated urothelial cells of the exposed workers (n = 30). Mutagenicity data were calculated after adjustment for urinary creatinine concentrations and ln-normalized; other data are from reference 37. The two BZ-dye workers who had non-mutagenic urine (subjects 121 and 128) were assigned mutagenicity values of 2.75 rev/µmol creatinine as described in the legend to Figure 2.

The correlation between urinary mutagenicity and the level of the presumptive C8dG-ABZ DNA adduct in urothelial cells was determined using the data from 30 subjects with confirmed BZ exposure: 15 BZ-dye workers and 15 BZ workers. As shown in Figure 5, a strong, positive correlation was found between urinary mutagenic potency and the level of the DNA adduct in urothelial cells (Pearson r = 0.59, $P <$ 0.0006). As reported previously (37), there was also a strong and highly significant positive correlation between the level of urinary

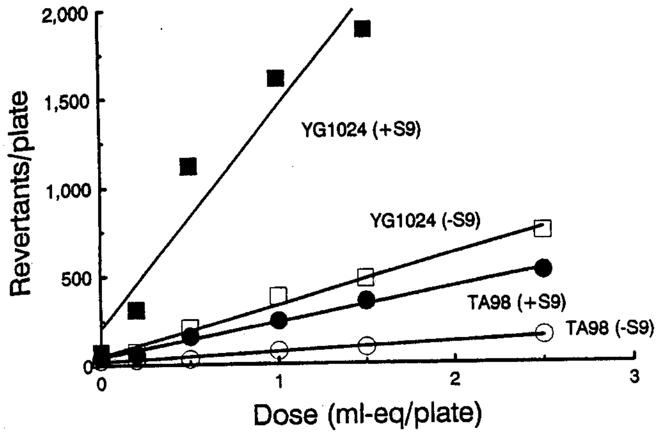

Fig. 6. Mutagenicity dose–response curves of the C18/methanol organic extract from a worker exposed to BZ (subject 141) in strains TA98 and YG1024 of *Salmonella* in the presence or absence of aroclor-induced rat liver S9. The mutagenic potencies (rev/ml-eq) are 52 (TA98, –S9), 194 (TA98, +S9), 283 (YG1024, –S9), and 1248 (YG1024, +S9).

metabolites and the level of the DNA adduct in urothelial cells among 33 exposed workers (Pearson $r = 0.68$, $P < 0.0001$).

Role of acetylation

In order to explore the role of acetylation in the urinary mutagenicity studied here, the urine from a BZ worker (subject 141) was evaluated in *Salmonella* strains TA98 and YG1024 in the presence and absence of S9. Only a small amount of urine remained after our initial studies, and subject 141 was chosen because the small amount remaining was sufficient to generate additional dose–response curves due to the relatively high mutagenic potency of this sample. The results (Figure 6) show that the urine of this BZ worker had direct-acting mutagenic activity in TA98 (52 rev/ml). The addition of enhanced acetylation (TA98, –S9 versus YG1024, –S9 or TA98, +S9) resulted in a 4- to 5-fold increase in the mutagenic potency of the urine, indicating that some acetylated metabolites are more mutagenic than their non-acetylated forms. The BZ worker's urine exhibited a 24-fold increase in mutagenic potency in strain YG1024 in the presence of S9 (acetylation and *N*-hydroxylation) compared with TA98 in the absence of S9 (minimal acetylation and no *N*-hydroxylation). This suggests that the combination of these two enzymatic activities results in the production of (i) a variety of mutagenic species and/or (ii) highly potent species of BZ in the urine, which are probably metabolites that are both acetylated and *N*-hydroxylated.

Discussion

Urinary mutagenicity as a general biomarker

Urinary mutagenicity provides an integrated measure of genotoxic exposure because it detects an effect due to a range of contributors to genotoxicity, not just those agents that are specific to the exposure being studied. In the present study, all but two of the controls had mutagenic urine, although none had detectable levels of BZ, ABZ, or DABZ in their urine (37); the detection limit for these three compounds was 500, 800, and 1500 ng/ml, respectively (56). Thus, agents other than BZ were probably responsible for the urinary mutagenicity observed in the control group.

Cigarette smoking can contribute to urinary mutagenicity, and previous studies (29,59,60) and our own observations (unpublished) show that the mutagenic potency of the urine from non-smokers is <5 rev/ml-eq in strain YG1024 (+S9), whereas the urine from smokers' produces ~20–40 rev/ml or ~2–4 rev/µmol creatinine. The mean ± SE urinary mutagenicity among the controls in this study was 17.1 ± 3.2 rev/ml-eq or 2.8 ± 0.7 rev/µmol creatinine, which is within the range of smokers' urine. Although cigarette-use was negligible among the workers in this study, bidi-use was common and was strongly associated with urinary mutagenicity among the 13 controls (see Results). Thus, ~47% of the variance (R^2) in urinary mutagenicity among the controls can be explained by bidi-use. In contrast, among the 30 exposed workers, bidi-use was not associated with urinary mutagenicity after adjusting for metabolite levels (see Results). Thus, BZ-associated metabolites alone accounted for 78% of the variance in urinary mutagenicity among the exposed workers, overwhelming any potential contribution from bidi-use among this group.

Similar to the urinary mutagenicity data, and in contrast to the metabolite data, all of the controls also had low, but detectable levels of the presumptive C8dG-ABZ DNA adduct in their exfoliated urothelial cells (37). This suggests that non-BZ-related DNA adducts were probably detected among the controls; however, the levels of DNA adducts among both the controls and exposed workers were not associated with smoking bidis (data not shown). As discussed above, urinary mutagenicity and DNA adducts detected by the ^{32}P-postlabeling assay are biomarkers that can integrate exposure to a variety of genotoxic agents into a single measurement. Thus, it is interesting that both of these assays, as opposed to the metabolite analysis, detected low, but measurable genotoxic endpoints in the controls. This illustrates the value of including a general biomarker such as urinary mutagenicity into biomonitoring/biomarker studies. Such a biomarker provides the opportunity to detect the potential contribution of other genotoxins in addition to those that are the focus of the study.

Comparison with other urinary mutagenicity studies

The mutagenic potencies of the urines studied here are difficult to compare with those of other subjects in the literature because no other study has used the combination of extraction method (C18/methanol) and *Salmonella* strain (YG1024) used here. However, three studies of smokers' urine that used strain YG1024 (in combination with either Blue Rayon/methanol/ammonia or XAD-2/acetone to extract the urinary organics) found that smokers' urine produced ~20–40 rev/ml or ~2–4 rev/µmol creatinine (29,59,60). These values for smokers are similar to those of the BZ-dye workers who had a mean of 39.6 rev/ml or 4.5 rev/µmol creatinine. Thus, the BZ-dye workers, who had negligible levels of either smoking cigarettes or bidis, had urinary mutagenicity values similar to those of smokers, who are another group at risk for bladder cancer from exposure to arylamines (31). The urinary mutagenicity of people who eat fried meat (due to heterocyclic amines) in strain TA98 can also be similar to that of smokers and, by extension, the BZ-dye workers, depending on how cooked the meat is and how much is consumed (13–15).

The only previous study of urinary mutagenicity among workers presumptively exposed to BZ or BZ-dyes used *Salmonella* strain TA98 and found no difference between a group of textile workers (exposed group) and a control group using urine extracted by XAD2–2/acetone (21). However, a significant difference was found between controls and exposed workers when the urines were first incubated with β-glucuronidase. The failure of that study, in contrast to ours, to find differences in the urinary mutagenicity between the controls and presumptively exposed workers in the absence of β-glucuronidase suggests that the methods used were insensitive and/or the exposures were low.

In this regard, a recent study (33) found that the urine of cigarette smokers extracted by C18/acetone was as much as four times more mutagenic than the same urines extracted by XAD-2/acetone, which was the method used in the textile study (21). Considering that arylamines contribute substantially to the mutagenic activity of smokers' urine (61), and our own finding that C18/methanol permitted the recovery of ~100% of BZ in water, XAD-2 may not have been a suitable extraction resin for the textile workers' urine. Also, the presumptive exposure of the textile workers to arylamines was not assessed in the other study by any additional methods, such as measurements of BZ in the air or BZ metabolites in the urine.

Correlation of urinary mutagenicity with other biomarkers

The correlation obtained here between urinary mutagenicity and urinary metabolites (r = 0.88) is the highest correlation ever found between these two biomarkers for exposure to any mutagenic agent, which includes trinitrotoluene, smoking, and coal tar (24,28,31–35). This may reflect the nature of the exposure itself (BZ and BZ-dyes), the potency of these compounds as genotoxic bladder carcinogens, as well as the sensitivities of the organic extraction/mutagenicity assay and metabolite analyses used in the present study. The correlation between urinary mutagenicity and urothelial DNA adduct levels (r = 0.59) is similar to the correlations found between urinary mutagenicity and either DNA adducts or hemoglobin adducts in cigarette smokers (28,32,36). The correlation between urinary metabolites and urothelial DNA adduct levels (r = 0.68) is similar to the correlations found between the urinary levels of cotinine/nicotine and DNA or hemoglobin adducts in smokers (28,32) and between a specific aflatoxin–DNA adduct and urinary metabolite (aflatoxin M1) in people exposed to aflatoxin (4).

Although the high correlation between urinary mutagenicity and urinary metabolites is notable, the correlation between urinary mutagenicity and urothelial DNA adduct levels is, perhaps, most interesting. In addition to cigarette smoking (32,36), the present study on workers exposed to BZ describes the only other exposure in which such a correlation has been examined. The relationship between a DNA adduct and the mutations that are generated by a cell due to the presence of that adduct is complex (62). Although the present data are only correlative, it is probable that the same DNA-reactive BZ metabolites in the urine that resulted in the detected urinary mutagenicity also formed the urothelial cell DNA adducts detected by ^{32}P-postlabeling and would, presumably, present an elevated risk for mutagenesis in the bladder epithelium.

Mutagenic forms of benzidine in human urine

As shown previously for two BZ-dye and two BZ workers evaluated in this study (56), the urinary concentrations of ABZ were generally many-fold greater than those of BZ or of DABZ, and >90% of the measured compounds were present in an acetylated form, i.e. were ABZ and/or DABZ (37). These findings are consistent with those of a previous study of workers exposed to BZ-based dyes (63) and to results from *in vitro* studies using human liver S9 (64) and fresh human liver slices (42). Urine from either smokers or non-smokers from the general population who are not otherwise exposed to genotoxic agents has essentially no mutagenic activity in TA98 in the absence of S9 (14–16,20). Our finding that urine from a BZ worker was mutagenic in strain TA98 in the absence of S9 (Figure 6) indicates that electrophilic metabolites (presumably BZ-associated) were present in this urine sample. Although DABZ is not a direct-acting mutagen in *Salmonella* TA1538 (which is similar to TA98 except that it lacks the pKM101 plasmid), a likely candidate is *N*-OH-DABZ, which is a direct-acting mutagen in strain TA1538 (65).

The 4- to 5-fold increase in mutagenicity of the BZ worker's urine in the presence of enhanced acetylation (Figure 6) is consistent with the finding that additional acetylation of *N*-OH-DABZ (achieved by adding *N,O*-acetyltransferase to *N*-OH-DABZ in the Salmonella assay) resulted in a 6-fold increase in mutagenicity compared with *N*-OH-DABZ itself (65). The 24-fold increase in mutagenic potency of the BZ worker's urine in the presence of enhanced acetylation and *N*-hydroxylation (Figure 6) is consistent with the production of highly mutagenic species that are both acetylated and *N*-hydroxylated. Collective evidence from Salmonella, mammalian cells, and rodents supports the view that C8dG adducts of *N*-OH-ABZ, for example, are responsible for some of the mutagenic and cytogenetic effects of BZ (66–68).

In this regard, studies in an acetylating strain of Salmonella have shown that BZ is three times more mutagenic than ABZ, indicating that mono-acetylated BZ (ABZ) is more mutagenic than di-acetylated BZ (DAZB) (69). Although ABZ may be three times more mutagenic than DABZ, the finding that >90% of the metabolites detected in the urine of the workers in this study were in an acetylated form (37) suggests that much of the mutagenic activity detected in our study (YG1024 +S9) may have been due to the production by YG1024 and S9 of *N*-OH-DABZ.

The mutagenicity of BZ has been studied in transgenic strains of *Salmonella* containing either human NAT1 or NAT2 (70), and a recent *in vitro* study using human recombinant NAT1 and NAT2 alleles and human liver slices found that BZ and ABZ were preferred substrates for NAT1 (71). However, previous analysis of the workers in the present study found no influence of various NAT1 and NAT2 polymorphisms on the levels of urinary metabolites or urothelial DNA adducts (37).

Conclusions

Although there are few workers remaining world-wide who are exposed to BZ or BZ-dyes, BZ and BZ-related compounds are still present in the environment. BZ is present in hazardous waste sites on the US EPA National Priorities List, ranks 49th of 275 on the 1994 'Priority List of Hazardous Substances' of the Agency for Toxic Substances and Disease Registry (72), and may contribute to the mutagenicity of waste-water effluent discharged by the dye industry (73). In addition, BZ can be activated to stable, mutagenic forms by green plants, providing a mechanism for the translocation of activated forms of BZ through the food chain (74). Nonetheless, BZ as a contributor to human bladder cancer is now largely eliminated due to the closure of factories that made or used BZ-related compounds.

The BZ-related exposures studied here have provided a model system in which a variety of biomarkers could be determined and compared. The ability to distinguish a low-exposure group from controls is a valuable feature of a biomarker, and this was demonstrated here by urinary mutagenicity and urinary metabolite analysis. All three biomarkers studied here distinguished between the controls and the BZ workers and correlated strongly with each other. The especially strong, positive correlation between urinary mutagenicity and urinary metabolites suggests that the methods used in these analyses were highly sensitive and detected the same or related compounds. The controls, whose urine did not contain any of the BZ-related compounds analyzed, did have detectable levels of urinary mutagenicity and urothelial DNA adducts. This illustrates the ability of such biomarkers to provide an integrated measure of genotoxic exposure, permitting the identification of additional contributors to the genotoxicity measured in both the control and exposed groups.

This study confirms that a general biomarker that is relatively inexpensive and simple to measure, such as urinary mutagenicity, can distinguish a low-exposure group from controls and can correlate strongly with other biomarkers of exposure. Such a biomarker may be of value for biomonitoring when specific biomarkers do not exist or are not cost-effective and to (i) detect uncharacterized genotoxic exposures among the controls, (ii) identify low-dose exposures, and (iii) characterize the chemical nature of urinary mutagens. This study demonstrates the advantages and limitations of the biomarkers evaluated here and should provide guidance in the selection and inclusion of such biomarkers into specific epidemiological study designs (2).

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